APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1992, p. 696–698
0099-2240/92/020696-03$02.00/0

Vol. 58, No. 2

NOTES

Potential Early Intermediates in Anaerobic Benzoate Degradation by *Rhodopseudomonas palustris*

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Received 5 August 1991/Accepted 13 November 1991

Alkali-treated extracts of *Rhodopseudomonas palustris* growing photosynthetically on benzoate were examined by gas chromatography/mass spectrometry for partially reduced benzoate derivatives. Two cyclic dienes, cyclohexa-2,5-diene-1-carboxylate and cyclohexa-1,4-diene-1-carboxylate, were detected. Either compound supported cell growth as effectively as benzoate. These results suggest that these cyclohexadienecarboxylates, probably as their coenzyme A esters, are the initial reduction products formed during anaerobic benzoate metabolism by *R. palustris*.

The degradation of aromatic compounds in anaerobic systems follows pathways distinct from the well-studied aerobic pathways, which all involve oxygenases. In classic experiments, Dutton and Evans (2) incubated anaerobic suspensions of the nonsulfur phototrophic bacterium *Rhodopseudomonas palustris* with radioactive benzoate in the presence of potential metabolites. They recovered radioactive cyclohex-1-ene-1-carboxylate, cyclohexanecarboxylate, and pimelate, which led them to propose a reductive, rather than oxidative, pathway leading to ring opening. However, they did not test any cyclohexadienecarboxylates, and they could not detect benzoate metabolism in cell extracts. Thus, the initial reduction products in *R. palustris* or in any other organism able to grow anoxically on aromatic compounds have not been determined (for a review, see reference 4).

In *R. palustris*, it is clear that the reductive process is preceded by coenzyme A (CoA) thioesterification of benzoic acid, enabling the cells to accumulate an otherwise permeant molecule (7), and that β-oxidation of the fully reduced cyclohexanecarboxylate occurs at the CoA ester level (9). It thus seems likely that other intermediates on the reductive pathway are also CoA esters. We have therefore examined for partially reduced benzoate derivatives the fraction of benzoate-grown *R. palustris* cultures that becomes ether soluble only after mild treatment with alkali; this fraction should include the carboxylic acids released upon hydrolysis of CoA thioesters. Gas chromatography/mass spectrometry (GC/MS) was chosen for the analysis because of its sensitivity and the relative speed and simplicity of sample preparation.

Cultures of *R. palustris* CGA 001 (7) were grown in PM, an inorganic salts medium (8) with 5 mM benzoate as the carbon source in completely filled 250-ml bottles at approximately 30°C, about 30 cm from a 25 W incandescent light bulb. When the benzoate concentration in the medium was still at least 1 mM, the culture was acidified by the addition of perchloric acid to 0.5 M. Under argon, cell debris was removed by centrifugation and free benzoic acid (e.g., from the medium) and any other acidic metabolites were extracted with diethyl ether. The aqueous layer was brought to pH 13 with KOH, placed in a 60°C water bath for 30 min, cooled, and filtered. The filtrate was acidified to pH 1 with HCl and extracted with three portions of ether. This ether extract was dried with $MgSO_4$, made alkaline with triethylamine, and evaporated to ~20 μl of oil. A 3-μl portion of the oil was methylated in 0.1 ml of 0.1 M diazomethane in ether and analyzed immediately on a Supelco SP2330 capillary GC column (catalog no. 2-4073M) mounted on a Finnigan 9610 gas chromatograph interfaced to a Finnigan 4500 quadrupole mass spectrometer. Methyl benzoate and three related alicyclic compounds were detected; each was identified by comparison of its retention time and mass spectrum with those of authentic standards. Methyl benzoate was by far the most abundant compound, but there was also a small peak (~8% as large as the methyl benzoate peak) identified as methyl cyclohex-3-ene-1-carboxylate. Significantly, traces of the methyl esters of cyclohexa-1,4-diene-1-carboxylate (≤0.2% as abundant as methyl benzoate) and cyclohexa-2,5-diene-1-carboxylate (~0.5% as abundant as methyl benzoate) were unequivocally identified by their retention times and mass spectra (Fig. 1). None of these alicyclic compounds was detected in the starting benzoic acid (as expected, since benzoic acid is manufactured by the oxidation of toluene [12]) under conditions in which deliberate 0.1% contamination was obvious; this implies that the bacteria made the alicyclic compounds. Because some of the methyl benzoate could represent medium-derived benzoic acid remaining after the first extraction, the intensities of the alicyclic ester peaks relative to methyl benzoate may represent minimum estimates of the intracellular ratios of the respective CoA species, assuming the recoveries of the different compounds were similar. Owing perhaps to differences in experimental design, we were unable to find methyl cyclohex-1-ene-1-carboxylate and methyl cyclohexanecarboxylate, even though the corresponding acids were labeled in Dutton and Evans' experiments (2). Analysis of cultures grown on fully ring-deuterated benzoate gave results similar

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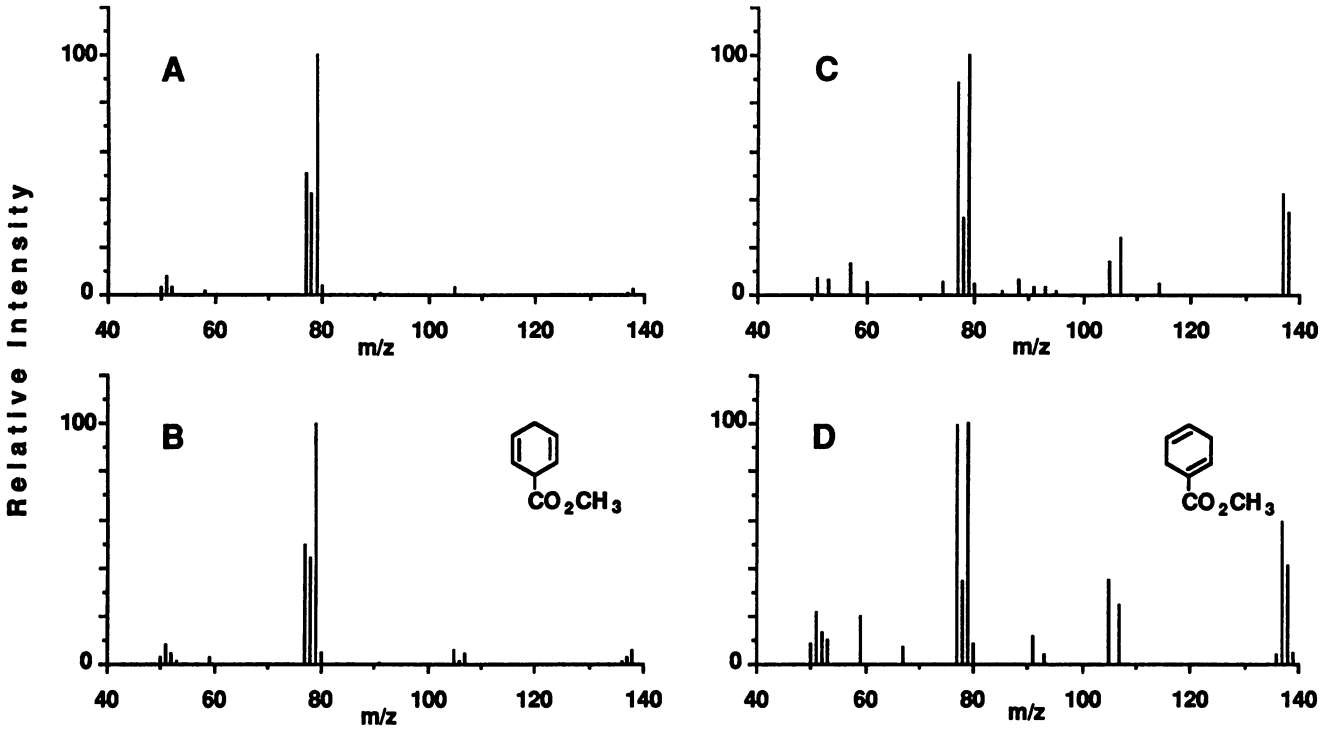

FIG. 1. Mass spectra of cyclohexadienecarboxylates. (A) Methyl cyclohexa-2,5-diene-1-carboxylate from a cell extract; (B) methyl cyclohexa-2,5-diene-1-carboxylate standard; (C) methyl cyclohexa-1,4-diene-1-carboxylate from a cell extract; (D) methyl cyclohexa-1,4-diene-1-carboxylate standard.

to those described above, except that the fragments in the mass spectra appeared at *m/z* values 5 U higher than before. This confirms that the alicyclic compounds were derived from benzoate and also shows that their formation was not accompanied by significant exchange of the ring hydrogens.

The experiments above were designed to bring the samples to analysis as quickly as possible, so as to maximize the chances of detecting small amounts of autoxidizable substances. However, the data do not provide a measure of intracellular metabolite concentrations, and they only suggest, but do not prove, that metabolites were present as CoA esters. Therefore, the perchloric acid-soluble fraction was examined (after titration to pH 3 with KOH, filtration, and lyophilization) for intact CoA esters by high-pressure liquid chromatography. A significant base-sensitive peak comigrating with benzoyl CoA was detected. Integration of this peak and measurement of the protein content of the culture gave an approximate benzoyl CoA content of 1 nmol/mg of protein, corresponding to an intracellular concentration of ~200 μM (based on a value of ~5 μl [internal volume] per mg of protein, measured as described previously [7]). A peak comigrating with the CoA ester of cyclohex-3-ene-1-carboxylic acid (commercially unavailable CoA esters were made essentially as described by Stadtman [11]; where used, anhydrides were made by reaction of the acid with a 0.5 equivalent of dicyclohexylcarbodiimide in chloroform) had about 7% the area of the benzoyl CoA peak, consistent with an intracellular concentration of ~15 μM. On the other hand, despite the availability of authentic standards, the CoA esters corresponding to the cyclohexadienecarboxylates were not identified. Since the GC/MS data suggest that the cyclohexadienecarboxylates were at least 10-fold less abundant than cyclohex-3-ene-1-carboxylate, the lower sensitivity of high-pressure liquid chromatography and the longer sample preparation time, rather than the nonexistence of the compounds, could account for this failure. Taken together, these analyses indicate that the cyclohexadienecarboxylates may be present in relatively low steady-state concentrations in the cells even though benzoyl CoA is abundant. This, in turn, suggests that the initial reduction may be the rate-limiting step in the reductive pathway.

We have also found that *R. palustris* grew well on either cyclohexa-1,4-diene-1-carboxylate (made as described previously [3]) or cyclohexa-2,5-diene-1-carboxylate (made as described previously [10]) as the sole carbon source for photosynthetic growth, which suggests that both compounds are metabolically relevant. This contrasts with the report that a *Moraxella* sp. grew on cyclohexa-2,5-diene-1-carboxylate but not on the 1,4 isomer (13). The observed doubling times of 15 to 17 h were close to those achieved on benzoate, and the molar growth yields were comparable. It is possible that the normal complement of benzoate-utilizing enzymes in *R. palustris* suffices for metabolism of exogenous cyclohexadienecarboxylates; two previously described aromatic acid CoA ligases (5, 6) each utilized either alicyclic acid as an alternate substrate. Under standard assay conditions (6), initial rates were 60 to 80% of those observed with benzoate.

The presence in cells of metabolizable compounds derived from benzoate does not prove that these compounds are true intermediates, rather than by-products, in benzoate degradation; detailed genetic and enzymological studies will be required to establish the pathway with confidence. Nonetheless, our experiments suggest that cyclohexa-2,5-diene-1-carboxylate and cyclohexa-1,4-diene-1-carboxylate, presumably as their CoA esters, are the first stable products in the reductive pathway in *R. palustris*. Each compound

corresponds formally to the addition of two hydrogen atoms to the benzoyl group, which loses the equivalent of one double bond in the process. Chemical reduction of benzoate to a nonconjugated diene is well known; cyclohexa-2,5-diene-1-carboxylate is obtained in ammonia-ethanol solution by using sodium as the reductant, an example of Birch reduction (1), although the enzymatic and chemical reactions need not be mechanistically related. It is also not clear why both cyclohexa-2,5-diene-1-carboxylate and cyclohexa-1,4-diene-1-carboxylate were present. It is possible that one isomer is the initial reduction product and that the cells convert it into the other isomer before carrying out further reduction, e.g., to a cyclohexenecarboxylate CoA ester. Alternatively, a single enzyme that disrupts an aromatic ring with over 30 kcal of resonance energy per mol might produce a mixture of products. In any event, characterization of the enzyme(s) and cofactor(s) involved in the first reductive step(s) in anaerobic benzoate metabolism will enlarge our understanding of the chemical capabilities of biological systems.

We appreciate the skilled work of mass spectroscopist Arthur Fogiel and the assistance of Ivan M. Turner. We also thank Benito V. Marrufo for carrying out the growth experiments. This work was supported in part by a grant (DE-FG02-86ER13495) to J.G. from the U.S. Department of Energy.

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